

Applicants: Short and Keller  
Application No.: 09/848,095  
Filed: May 3, 2001  
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In the Specification

Please enter the Abstract amended to read as follows:

A1  
Disclosed is a process for identifying clones having a specified activity of interest, which process comprises (i) generating one or more expression libraries derived from nucleic acid directly isolated from the environment; and (ii) screening said libraries utilizing a fluorescence activated cell sorter to identify said clones. More particularly, this is a process for identifying clones having a specified activity of interest by (i) generating one or more expression libraries derived from nucleic acid directly or indirectly isolated from the environment; (ii) exposing said libraries to a particular substrate or substrates of interest; and (iii) screening said exposed libraries utilizing a fluorescence activated cell sorter to identify clones which react with the substrate or substrates. Also provided is a process for identifying clones having a specified activity of interest by (i) generating one or more expression libraries derived from nucleic acid directly or indirectly isolated from the environment; and (ii) screening said exposed libraries utilizing an assay requiring co-encapsulation, a binding event or the covalent modification of a target, and a fluorescence activated cell sorter to identify positive clones.

Please amend Paragraph [0147] beginning on page 41 to read as follows:

A2  
(Amended) Several methods have been described for using reporter genes to measure gene expression. These reporter genes encode enzymes not ordinarily found in the type of cell being studied, and their unique activity is monitored to determine the degree of transcription. Nolan *et al.*, developed a technique to analyze  $\beta$ -galactosidase expression in mammalian cells employing fluorescein-di-( $\beta$ -D-galactopyranoside (FDG) as a substrate for  $\beta$ -galactosidase, which releases fluorescein, a product that can be detected by a fluorescence-activated cell sorter (FACS) upon hydrolysis (Nolan *et al.*, 1991). A problem with the use of FDG is that if the assay

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is performed at room temperature, the fluorescence leaks out of the positively stained cells. A similar problem was encountered in other studies of  $\beta$ -galactosidase measurements in mammalian cells and yeast with FDG as well as other substrates (Nolan *et al.*, 1988; Wittrup *et al.*, 1988). Performing the reaction at 0°C appreciably decreased the extent of this leakage of fluorescence (Nolan *et al.*, 1988). However this low temperature is not adaptable for screening for, for instance, high temperature  $\beta$ -galactosidases. Other fluorogenic substrates have been developed, such as 5-dodecanoylamino fluorescein di- $\beta$ -D-galactopyranoside (C<sub>12</sub>-FDG) (Molecular Probes) which differs from FDG in that it is a lipophilic fluorescein derivative that can easily cross most cell membranes under physiological culture conditions. The green fluorescent enzymatic hydrolysis product is retained for hours to days in the membrane of those cells that actively express the *lacZ* reporter gene. In animal cells C<sub>12</sub>-FDG was a much better substrate, giving a signal which was 100 times higher than the one obtained with FDG (Plovins *et al.*, 1994). However in Gram negative bacteria like *E. coli*, the outer membrane functions as a barrier for the lipophilic molecule C<sub>12</sub>-FDG and it only passes through this barrier if the cells are dead or damaged (Plovins *et al.*). The fact that C<sub>12</sub> retains FDG substrate inside the cells indicates that the addition of unpolarized tails may be used for retaining substrate inside the cells with respect to other enzyme substrates.

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Please amend Paragraph [0148] on page 42 to read as follows:

A3 (Amended) The abovementioned  $\beta$ -galactosidase assays may be employed to screen single *E. coli* cells, expressing recombinant  $\beta$ -D-galactosidase isolated from a hyperthermophilic archaeon such as *Sulfolobus solfataricus*, on a fluorescent microscope. Cells are cultivated overnight, centrifuged and washed in deionized water and stained with FDG. To increase enzyme activity, cells are heated to 70°C for 30 minutes and examined with a fluorescence phase contrast microscope. *E. coli* cell suspensions of the  $\beta$ -galactosidase expressing clone stained with C<sub>12</sub>-FDG show a very bright fluorescence inside single cells (Figure 8).

Please amend Paragraph [0185] on page 54 to read as follows:

A4 (Amended) Probe nucleic acid sequences designed according to the method described above can also be utilized in the present invention to "enrich" a population for desirable clones. "Enrich", as utilized herein, means reducing the number and/or complexity of an original population of molecules. For example, probes are designed to identify specific polyketide sequences, and utilized to enrich for clones encoding polyketide pathways. Fosmid libraries are generated in *E. coli* according to the methods described in the Example herein. Clones are encapsulated and grown to yield encapsulated clonal populations. Cells are lysed and neutralized, and exposed to the probe of interest. Hybridization yields a positive fluorescent signal which can be sorted on a fluorescent cell sorter. Positives can be further screened via expression, or activity, screening. Thus, this aspect of the present invention facilitates the reduction of the complexity of the original population to enrich for desirable pathway clones. These clones can be utilized for further downstream screening. For example, these clones

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can be expressed to yield backbone structures (defined herein), which can then be decorated in metabolically rich hosts, and finally screened for an activity of interest. Alternatively, clones can be expressed to yield small molecules directly, which can be screened for an activity of interest. Further more, multiple probes can be designed and utilized to allow "multiplex" screening and/or enrichment. "Multiplex" screening and/or enrichment as used herein means that one is screening and/or enriching for more than desirable outcome, simultaneously.

Please amend Paragraph [0189] on page 56 to read as follows:

A5  
(Amended) *Streptomyces venezuelae*, unlike most other *Streptomyces* species, has been shown to sporulate in liquid growth culture. In some media, it also fragments into single cells when the cultures reach the end of vegetative growth. Because the production of most secondary metabolites, including bioactive small molecules, occurs at the end of log growth, it is possible to screen for *Streptomyces venezuelae* fragmented cells that are producing bioactivities by a fluorescence analyzer, such as a FACS machine, given the natural fluorescence of some small molecules.

Please amend Paragraph [0194] on page 57 to read as follows:

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(Amended) In the method of the present invention, the fluorescing properties of this and other similar compounds are utilized to screen for compounds of interest, as described previously, or are utilized to enrich for the presence of compounds of interest. Host cells expressing recombinant clones potentially encoding gene pathways are screened for fluorescing properties. Thus, cells producing fluorescent proteins or metabolites can be identified. Pathway clones expressed in *E.coli* or other host cells, can yield bioactive compounds or "backbone

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structures" to bioactive compounds (which can subsequently be "decorated" in other host cells, for example, in metabolically rich organisms). The "backbone structure" is the fundamental structure that defines a particular class of small molecules. For example, a polyketide backbone will differ from that of a lactone, a glycoside or a peptide antibiotic. Within each class, variants are produced by the addition or subtraction of side groups or by rearrangement of ring structures ("decoration" or "decorated"). Ring structures present in aromatic bioactive compounds are known in some instance to yield a fluorescent signal, which can be utilized to distinguish these cells from the population. Certain of these structures can also provide absorbance characteristics which differ from the background absorbance of a non-recombinant host cell, and thus can allow one to distinguish these cells from the population, as well. Recombinant cells potentially producing bioactive compounds or "backbone" structures can be identified and separated from a population of cells, thus enriching the population for desirable cells. Thus, the method of the present invention also facilitates the discovery of novel aromatic compounds encoded by gene pathways, for example, encoded by polyketide genes, directly from environmental or other samples.